Norethindrone ensures masculinization, normal growth and secondary sexual characteristics in the fighting fish, *Betta splendens*

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To ensure masculinization and normal growth of the obligately air-breathing fighting fish *Betta splendens*, norethindrone acetate (NE) was administered through discrete immersions for 3 h each on the second, fifth and eighth day post-hatching (dph) at selected concentrations (250, 500, 750, 1000 and 1250 μg/l). Immersions at 1000 μg/l ensured the maximum of 92% masculinization, 71% survival, normal growth and appearance of secondary sexual characteristics. NE reduced the air-breathing frequency on the fifth and eigth dph. It also reduced the frequency in males depurated for 172 days following the treatment, although males treated at 750 μg/l almost restored the frequency. In the ovary of the treated but persisting females, it reduced the number of vitellogenic oocytes and increased the vacuolar area. In the testis of the treated males, it reduced the number of spermatogonia and increased the vacuolar area. The treated males could neither induce the female to spawn as many eggs nor accommodate as many fertilized eggs within the bubble nest, as that of the control. While the control females attained puberty on the 140th dph and spawned 122 eggs once every 16 days, the persisting females, which were previously treated at 1000 μg/l, postponed puberty to the 183rd dph and prolonged the inter-spawning period to 40 days. During the 240-day experiment, NE reduced the cumulative progeny production from 764 to 104 (13.6% of the control).

Keywords: Air-breathing frequency, bubble nest, discrete immersions, suppressed growth.

IN recent years, culture and trade of ornamental fishes have a larger share in the aquaculture industry. With the well-developed veil tail, the males of the fighting fish *Betta splendens* Regan, 1910, are more attractive and fetch a higher price than the females. A number of publications show that enormous efforts have been made to develop a simple and widely practicable technique to generate all male progenies in the fighting fish[1]. Of the 16 androgens tested, 17α-methyltestosterone (MT) is the most widely used hormone and has been tested in more than 25 species, including the fighting fish[2]. Discrete immersions for 3 h each for 3 days in 900 μg MT/l ensured 100% masculinization in the fighting fish. The MT immersions, however, reduced body length of the male to 50% of the control. When allowed to court, the 'stunted' male was not readily accepted by a normal female and when accepted, could induce its partner to spawn 50% eggs only[3]. Thus these males could neither produce as many progenies nor were as attractive as the untreated ones. Hence there is a need to search for a steroid which not only masculinizes the progenies, but also sustains normal body growth.

Varadaraj[4] showed that the oral administration of 3 μg norethinodrone acetate (NE)/g diet produced 100% male tilapia (*Oreochromis mossambicus*) fry, which were 40% heavier than the controls. Pandian and Sheela[2] have noted that protocols for hormone treatment are available for about 50 species, using one of the 31 steroids. However, only two publications are available on masculinization of fishes using NE[4,5]. On administration of 8 mg NE/kg diet for 40 days, Kavumpurath and Pandian[5] obtained 100% male progenies of the fighting fish, with loss of 36% of the treated fry. However, they did not test whether the administered NE was anabolic and accelerated the body growth. Hence it is proposed to generate all male fighting fish progenies using the milder discrete immersion technique with lower dose of NE and to confirm whether these male progenies ensure normal body growth and secondary sexual characteristics. On exposure to the endocrine disrupting chemicals (EDC) like endosulfan, the gills of an air-breathing murrel *Channa punctatus*, being the first organ to come in contact with the EDC, are more sensitive than the kidney[6]. On being immersed, an obligate air-breathing fish like the fighting fish may escape to the surface to breath atmospheric air and not expose the gills to NE. Thus, another reason for selecting the fighting fish is to understand the effect of NE on the air-breathing frequency.

Materials and methods

Experimental fish

Healthy *B. splendens* were purchased from S. Ram Fish Farm, Madurai in spring 2003. They were reared (at

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26 ± 1°C; 14 L : 10 D) in large, circular aquaria (150 diameter × 180 H cm) containing well-aerated water (5.5 mg O_2/l) and aquatic plants. From natural spawning in the bubble nest, fertilized eggs were collected and allowed to develop in a glass bowl (1 l). Hatchlings were transferred to nursery aquaria (4 l) at a density of 100–120 hatchlings/aquarium and fed to satiation initially with paramecium and granules of boiled egg, and subsequently with *Artemia nauplii*, twice daily for three weeks. The fry were then transferred to larger aquaria (5 l) and fed pellet feed until they attained sexual maturity.

Discrete immersions

As against chronic exposure[7], the discrete immersion technique aims at the immersion(s) of the steroid-sensitive embryonic or hatchling stage in minimum water containing the chemical(s) to induce the desired sex reversal in fish[8,9]. As it is cost-effective, allows limited handling and reduces pollution, the discrete immersion technique was adopted[2].

To study the effect of NE (Sigma, St Louis, MO) on permanent masculinization of the fighting fish, randomly selected fry were subjected to discrete immersions for a certain duration (3 h/day) each at the second, fifth, and eighth day post-hatching (dph) in water containing selected dose of NE. Stock solution of NE was prepared in absolute alcohol at a concentration of 1 mg/ml. Aliquot of stock solution was added appropriately to a glass bowl containing 1 l of water to get the final steroid concentration for each treatment group accordingly. Hormonal treatment was carried out by transferring the fry to the glass bowl containing steroid-mixed water. Five groups of 40 fry each were subjected to discrete immersion at NE concentrations of 250, 500, 750, 1000 and 1250 µg/l water, selected based on preliminary experiments. There were five replicates for each treatment group. An untreated group served as control and another group immersed in water containing alcohol alone (sham-treated) served as the experimental control. Temperature in the aquarium water was maintained at 26 ± 1°C and photoperiod at 14 L : 10 D. Compressed air was gently forced through an air stone to provide adequate oxygenation and mixing.

Depuration

After completion of the hormone treatment, the fry in each group were separately reared in the aquarium (45*L* × 30*B* × 10*H* cm) and their survival, growth, sex ratio, and reproductive performance were monitored. Monthly growth measurements were made. For this, ten randomly selected individuals from each batch were anesthetized and kept in a glass tray, with a transparent graph (mm) sheet pasted onto the bottom. The body length of an individual fry was measured keeping the tray under a stereomicroscope (10X, Nikon, Japan).

Sex ratio

Sex was determined at juvenile stage at the age of 90–120 days by external examination of the dorsal, anal and caudal fins. The males have remarkably larger and more intensely coloured fins than the females. Sex was thus identified and the ratio was calculated.

Bubble nest

The pairs, randomly selected in each group, were allowed to breed in rectangular, transparent glass aquaria, each measuring 30*L* × 15*B* × 10*H* cm. Prior to commencement of courtship, the males blow bubbles to build a bubble nest, usually in a corner on the surface of water in the breeding tank. Following the procedure adopted by Jaroensutasinee and Jaroensutasinee[10], the length (*a*), width (*b*) and depth of the bubble nest were measured to the nearest 0.01 mm using a vernier calipers and its area (*A*) was estimated using the ellipsoid equation $A = \Pi ab$.

Sperm motility

Artificial stripping of milt is not possible in *B. splendens*. Hence from each group, five randomly selected, mature males were anaesthetized in 500 ml water containing 1 ml clove oil (Leo Pharma, India) and the gonads were dissected out. Then 2 µg tissue from one of the testes was minced for 1 min in 500 µl of Hank's saline[11]. On a microscopic slide with a well, 300 µl of tap water was added to 100 µl Hank's saline containing milt, and sperm motility duration was determined using a stopwatch (Shinco, India). The duration of motility was taken in seconds, when 50% of the sperm cells became immotile[12].

Fecundity

On attaining sexual maturity, the persisting female was paired with a normal male and the number of eggs in the bubble nest was counted. For each experiment, the female was allowed to court with three normal males.

Hatchability

The collected eggs (75–122); were thoroughly rinsed in clean water before being transferred into a glass bowl (100 ml). A glass pipette with an opening diameter of approximately 2 mm was used to handle the eggs and fry. After 24 h, unfertilized and arrested embryos were identified by the appearance of a milky-white colour and were removed from the bowl; the remaining developing eggs were observed until hatching. The hatchability was calculated by counting the number hatchlings as percentage of the total number of eggs.

Histology

One of the gonads was dissected out and directly mounted or fixed in 10% formalin. It was subsequently mounted in the tissue-freezing medium (Junk, Leica Instruments GmbH, Germany) at –17°C to obtain 6 μm thin sections in the freezing microtome (Minotome-Microtome Cryostat; International Equipment Company, Needham Heights, MA, USA).

These sections on the slide were fixed in methanol (100%) and kept overnight. Subsequently, the slide was rinsed with tap water followed by distilled water for 1 min and stained with haematoxylin and eosin, following the standard procedure (described by the manual catalogue card number 74-33828 of International Equipment Company, MA, USA). Then the sections were permanently mounted on the slide with DPX resin. They were scanned and photographed using a phase contrast microscope (Nikon Optiphot, Nikon Corp., Tokyo, Japan). Viewing under phase contrast microscope with objectives 10 and 100X, the vacuolar area in the ovary and testis, and spermatid density in the testis of the treated fish were estimated.

Air breathing

Being an obligate air-breather, *B. splendens* frequents the water surface for gaseous exchange. Earlier studies on monitoring the surfacing frequency of anabantids and channids have shown that the frequency is influenced by age[13] and depth of the aquarium[14]. To monitor air-breathing frequency of the fighting fish, two sets of observations were made. In the first one, the 2, 5 and 8-day-old fry were observed. This included five glass bowls, each containing equal volume of (100 ml) water + NE with a column height of 5 cm. The fry belonging to the control, experimental control and all the groups in the treated series were individually observed for a period of 30 min, twice a day (10 a.m. and 10 p.m.); previous observations lasting for periods of 1 and 2 h suggested that the 30 min observation was adequate. The number of times the fish visited the surface was counted; these values were calculated for the number of visits/h.

The second set of observations were made on the 180th day, i.e. when the treated fish were depurated in clean waters for 172 days. Five glass aquaria (each 5 l) were filled with equal volume of water (each 4 l) with a column height of 20 cm. The surfacing frequency of the fish was observed for 30 min and estimated, as described above.

Secondary sexual characteristics

To a responding female, the male began to attract her with frequent display of erected paired and unpaired fins, and operculum (stage 2). Following the procedure adopted by Kirankumar and Pandian[3], the frequency of erection of unpaired fins of five males was counted, each for a period of 10 min.

Statistical methods

Data analyses were based on the mean ± SE. The chi-square test (χ^2) was used to test the null hypothesis of no difference of male : female sex ratio between control and treated groups. Multiple comparison test (one-way ANOVA) was performed to assess survival of the fry, air-breathing frequency, nest-building frequency, sperm-motility duration, hatchability, inter-spawning period, fecundity, cumulative fecundity and hatchling between control and treated series. All the statistical analyses were performed using Sigmastat ver. 2.0.

Results

Survival

After the three immersions for a cumulative period of 9 h on the second, fifth and eigth dph, 91% fighting fish survived at 250 μg NE/l, but only 53% at the highest tested super-optimal dose of 1250 μg/l (Table 1). At the optimal dose of 1000 μg/l, which ensured maximum of 92% masculinization, 71% of the treated group survived at the end of the treatment, but only 65% on the 120th dph, when sexual dimorphism was readily recognizable. At the highest tested dose of 1250 μg/l, none of the treated individuals survived up to the 120th dph. Hence the fighting fish was not treated at doses higher than 1250 μg/l to achieve 100% masculinization.

Air breathing

When discretely immersed in NE containing water in bowls with equal volume (100 ml) and depth (5 cm), the fry belonging to the control, experimental control and treated series hung to the surface during the entire period of immersion. However, the control and experimental control series breathed air 6–7 and 10–12 times/h on the fifth and eight dph, respectively (Table 2). These values decreased significantly to two times/h at the dose of 750 and 1000 μg/l on the fifth dph, indicating a significant decrease in the air-breathing frequency following the treatment.

On the 180th day, a control fighting fish visited the surface to breath air 22 times/h. This air-breathing frequency has also been decreased to nine times/h in the group treated with a dose of 1000 μg/l. Interestingly, both the 8-day-old fry and 180-day-old adults, which were previously treated at 750 μg/l, visited the surface to breath air almost as many times as their respective controls.

Growth

On the day of hatching, the fighting fish measured 3 mm in body length in the control and all the groups in the treated series. The early log phase of accelerated growth was sustained until the 90th–120th dph in both the series

Table 1. Effect of different doses of norethindrone acetate (NE) on survival and sex reversal of the fighting fish *Betta splendens*, which were previously immersed for 9 h, i.e. 3 h each on the second, fifth and eighth day post-hatching (dph). Each value represents the mean of five batches, each consisting of 40 fry

	Survival at						
	End of treatment*		Sexual dimorphism*		Sex distribution (no.)		Sex ratio**
Dose (μg/l)	(no)	(%)	(no)	(%)	♂	♀	♂ : ♀
0	37 ± 0.5	94 ± 1.3[a]	36 ± 0.3	90 ± 0.8[c]	17 ± 0.4	19 ± 0.4	0.47 : 0.53
250	36 ± 0.9	91 ± 2.2[a]	32 ± 0.8	81 ± 2.0[d]	24 ± 0.9	8 ± 0.4	0.75 : 0.25***
500	35 ± 0.5	87 ± 1.3[a]	29 ± 1.0	73 ± 2.6[d]	23 ± 0.9	6 ± 0.4	0.79 : 0.21***
750	35 ± 0.4	88 ± 0.9[a]	31 ± 0.5	79 ± 1.3[d]	27 ± 0.2	4 ± 0.4	0.87 : 0.13***
1000	28 ± 0.5	71 ± 1.2[b]	26 ± 1.4	65 ± 3.6[d]	24 ± 1.4	2 ± 0.2	0.92 : 0.08***
1250	21 ± 1.4	53 ± 3.5[b]	–	–	–	–	–

*All values are the means with standard error of the mean (± SE); values bearing superscripts 'b' and 'd' are significantly ($P < 0.05$) different from their respective controls 'a' and 'c'.
**Chi square (χ^2) test with Yates correction; ***indicates significant ($P < 0.001$) deviation from the expected 0.5 : 0.5 ratio of male to female.

Table 2. Effect of different doses of NE on the air-breathing frequency of the fighting fish, which were immersed for 3 h each on the second, fifth and eighth dph. Each value represents the mean for three individuals and each individual was observed for a period of 30 min

	Air-breathing frequency (times/h) on the			
Dose (μg/l)	Second day	Fifth day	Eighth day	180th day
Control	Hung to the surface	7 ± 0.9[a]	10 ± 0.9[c]	22 ± 0.9[e]
Experimental control	Hung to the surface	6 ± 0.3[a]	12 ± 0.9[c]	23 ± 0.7[e]
250	Hung to the surface	4 ± 0.3[b]	5 ± 0.3[d]	12 ± 0.7[f]
500	Hung to the surface	3 ± 0.3[b]	5 ± 1.2[d]	14 ± 0.7[f]
750	Hung to the surface	2 ± 0.9[b]	8 ± 1.2[c]	21 ± 1.5[e]
1000	Hung to the surface	2 ± 0.9[b]	3 ± 0.3[d]	9 ± 1.2[f]

All values are the means ± SE; Values bearing superscripts 'b', 'd' and 'f' are significantly ($P < 0.05$) different from their respective controls 'a', 'c' and 'e'.

(Table 3), but from the 120th dph, the control male grew significantly faster than the female and attained a longer body length of 47 mm on the 240th dph, while the female attained only 40 mm.

The observed differences in growth between the control and treated series became significant after the 120th dph in females (Table 3). Remarkably, the females attained body lengths of 36 mm and 35 mm at doses of 750 μg/l and 1000 μg/l on the 240th dph, while the control attained a body length of 40 mm, indicating that NE suppressed growth in the persisting females. Conversely, the males treated at sub-optimal doses of 500 and 750 μg/l grew faster and attained maximum body length until the 120th dph. For instance, those immersed at 750 μg/l attained a body length of 44 and 51 mm on the 120th and 240th dph respectively, compared to the control males attaining a body length of 36 and 47 mm respectively. Likewise, at the dose of 750 μg/l, the NE also sustained normal growth in the males until the 120th dph.

Sex ratio

With increasing NE dose of discrete immersions, the sex ratio was progressively biased towards males (Table 1). At the treatment dose of 1000 μg/l, a maximum of 92% males was generated. The secondary sexual characteristics of a treated male were similar in appearance (Figure 1) to that of the control male. For instance, the erected dorsal fin of the control and that treated at 500 μg/l measured 2 cm in length. When allowed to court, a treated male chased a normal female 15 ± 3 times within a period of 30 min, while a control male chased the female 17 ± 4 times within 30 min. Hence from the points of the secondary sexual characteristics and courtship behaviour, the treated males were as attractive and aggressive as the control males. Aggressive behaviour was estimated by counting the frequency of erection of dorsal, anal and caudal fins. For instance, the caudal fin erected 5 times every 10 min in the control and the group treated at 500 μg/l.

Female

Table 4 shows the significant negative reproductive effects of NE on the treated. but persisting females. (i) The age at which the first spawning occurred was postponed from the 140th dph in the control to the 183rd dph in those treated at 1000 μg/l. (ii) The inter-spawning period

Table 3. Effect of different doses of NE on growth of the fighting fish, whose fry were immersed for a cumulative period of 9 h, i.e. 3 h each on the second, fifth and eighth dph. Each value represents the mean growth of ten randomly selected fry each from five batches

	Body length (mm) on the						
Dose (μg/l)	30th day	60th day	90th day	120th day ♂	120th day ♀	240th day ♂	240th day ♀
Control	12 ± 0.9	18 ± 0.6^{a}	26 ± 1.2	36 ± 1.3^{cA}	32 ± 0.9^{eA_1}	47 ± 1.4^{gB}	40 ± 0.7^{iB_1}
250	10 ± 0.3	21 ± 0.6^{b}	28 ± 1.2	41 ± 1.3^{d}	32 ± 1.2^{e}	48 ± 1.2^{g}	38 ± 0.7^{i}
500	12 ± 1.2	21 ± 1.2^{b}	27 ± 1.4	44 ± 1.5^{d}	32 ± 1.2	51 ± 1.4^{h}	37 ± 1.4^{j}
750	11 ± 0.3	18 ± 0.9^{a}	25 ± 0.8	40 ± 0.9^{d}	28 ± 0.8^{f}	47 ± 0.5^{g}	36 ± 0.7^{j}
1000	11 ± 1.0	18 ± 1.0^{a}	24 ± 1.6	37 ± 1.8^{c}	26 ± 0.8^{f}	44 ± 0.7^{h}	35 ± 0.9^{j}
1250	10 ± 0.8	–	–	–	–	–	–

All values are the means ± SE; Values bearing superscripts 'b', 'd', 'f', 'h' and 'j' are significantly ($P < 0.05$) different from their respective controls 'a', 'c', 'e', 'g', and 'i'; Values without superscript letters are not significantly different from their respective controls; Values in selected rows bearing superscripts A and B are significantly ($P < 0.05$) different from their respective controls A_1 and B_1.

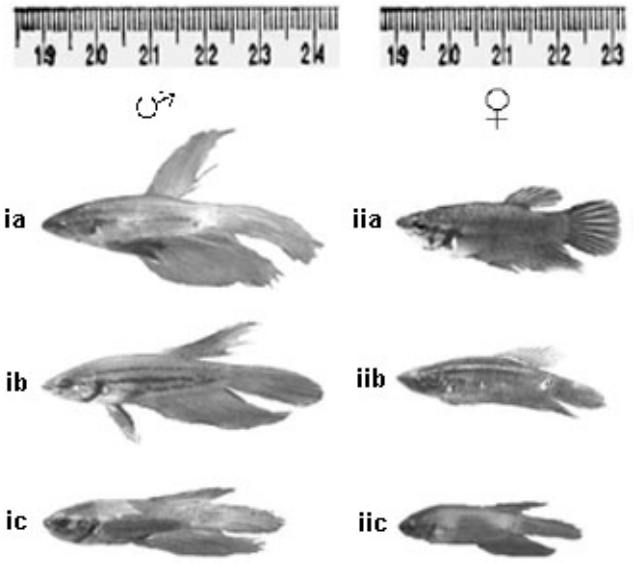

Figure 1. Effect of selected doses of norethindrone acetate iia–c, (NE) on body length attained by *Betta splendens* on the 240th dph. ia–c, Males; iia–c, Females. Row ia, iia, Control; ib, iib, 500 μg/l; dose and ic, iic, 1000 μg/l.

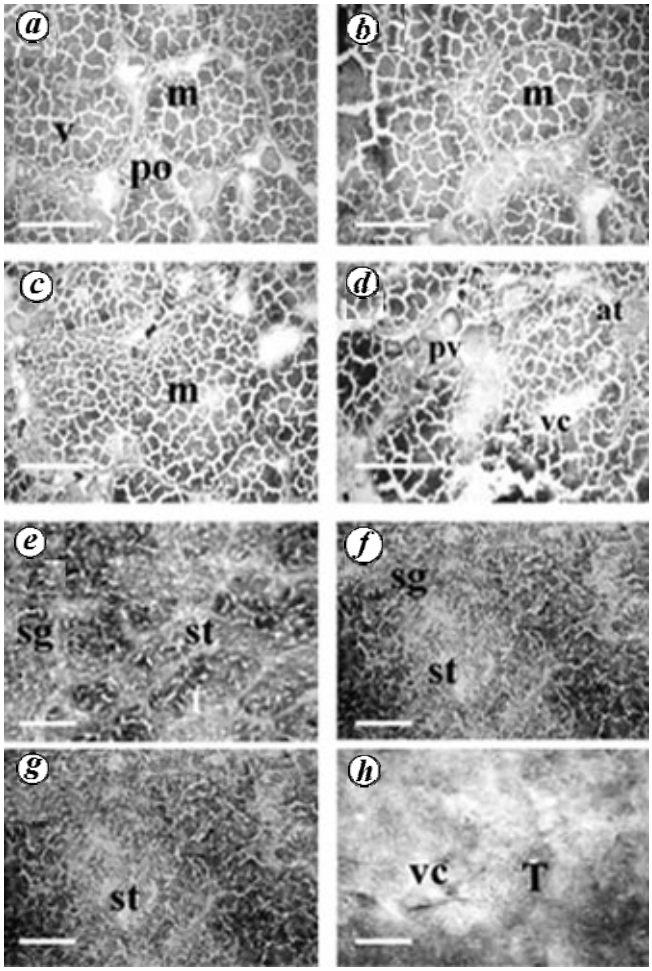

Figure 2. Effect of different doses of NE on the gonads of fighter fish. Ovary: (***a***) control; (***b***) 250 μg/l; (***c***) 500 μg/l and (***d***)1000 μg/l. po, Primary oocyte; pv, Pre-vitellogenic oocyte; v, Vitellogenic oocyte; vc, Vacuole; m, Matured oocyte; at, Atretic oocyte. Scale = 300 μm. Testis: (***e***) Control; (***f***) 500 μg/l; (***g***) 750 μg/l and (***h***) 1000 μg/l. sg, Spermatogonium; st, Spermatids; T, Seminiferous tubule. Scale = 25 μm.

was also prolonged from 16 to 40 days. (iii) The fecundity was reduced from 122 eggs/spawning to 75 eggs/spawning. (iv) The number of F_1 hatchlings was reduced from 110 to 53. During the 240-day experiment, the spawning frequency was significantly reduced from seven times in the control to two times in the females treated at 1000 μg/l (Table 4). Correspondingly, the cumulative fecundity was also reduced from 851 to 147 eggs. On the whole, the cumulative F_1 progeny production of the treated F_0 female was significantly reduced from 764 to 104, which is just 13.6% of the control. This is perhaps the first contribution to demonstrate the cumulative effects of NE on the treated but persisting female fish.

Figure 2 shows the degenerative changes that took place in the ovary of the treated series: (i) progressive reduction in the number of vitellogenic oocytes; for instance, when a section of the ovary of the control was

Table 4. Effect of different doses of NE on spawning and fecundity of the treated but persisting F_0 female fighting fish. These treated females were crossed with normal males. Each value represents the mean of five estimates.

Dose (μg/l)	Age at first spawning (dph)	Inter-spawning period (day)	Fecundity (no/spawning)	Hatchlings (no.)	Spawning frequency in 240 days (no.)	Cumulative fecundity (no)	Cumulative hatchlings (no.)
0	140 ± 3.1^{a}	16 ± 0.7^{c}	122 ± 1.9^{e}	110 ± 1.1^{g}	7 ± 0.2^{i}	851 ± 8.4^{k}	764 ± 7.5^{m}
250	163 ± 1.1^{b}	22 ± 1.3^{d}	104 ± 1.7^{f}	94 ± 2.2^{h}	4 ± 0.2^{j}	422 ± 7.8^{l}	381 ± 6.8^{n}
500	170 ± 2.6^{b}	24 ± 1.0^{d}	100 ± 2.8^{f}	89 ± 3.2^{h}	4 ± 0.2^{j}	401 ± 9.8^{l}	355 ± 8.7^{n}
750	172 ± 1.4^{b}	31 ± 1.5^{d}	88 ± 2.9^{f}	66 ± 2.0^{h}	3 ± 0.3^{j}	265 ± 9.8^{l}	197 ± 7.3^{n}
1000	183 ± 1.9^{b}	40 ± 1.2^{d}	75 ± 2.6^{f}	53 ± 2.3^{h}	2 ± 0.3^{j}	147 ± 4.9^{l}	104 ± 3.5^{n}

All values are the means ± SE; values bearing superscript 'b', 'd', 'f', 'h', 'j', 'l' and 'n' are significantly ($P < 0.05$) different from their respective control 'a', 'c', 'e', 'g', 'i', 'k' and 'm'.

Table 5. Effect of different doses of NE on the fighting fish, which were previously immersed for 9 h, i.e. 3 h each on the second, fifth and eighth dph after hatching. Each value represents the mean of five estimates. Age of maturity was fixed on the day when a male built its first bubble nest

Dose (μg/l)	Age of maturity (day)	Nest building frequency (days)	Motility duration (s)	Hatchability (%)
Control	110 ± 1.0^{a}	5 ± 0.2^{c}	103 ± 2.7^{e}	91 ± 1.0^{g}
250	116 ± 2.0^{a}	6 ± 0.4^{c}	96 ± 1.7^{e}	90 ± 1.3^{g}
500	91 ± 3.9^{b}	6 ± 0.2^{c}	96 ± 1.4^{e}	91 ± 0.5^{g}
750	160 ± 1.4^{b}	16 ± 0.5^{d}	93 ± 1.1^{f}	78 ± 1.3^{h}
1000	168 ± 1.3^{b}	28 ± 1.4^{d}	71 ± 2.4^{f}	66 ± 1.4^{h}

All values are the means ± SE; Superscripts 'b', 'd', 'f' and 'h' are significantly ($P < 0.05$) different from their respective controls 'a', 'c', 'e' and 'g'.

viewed under the phase contrast microscope with 10X objective, as many as nine vitellogenic oocytes were visible (Figure 2 *a*), but about five oocytes were recognizable in females treated at 1000 μg/l (Figure 2 *d*); (ii) obliteration and disappearance of the wall of the vitellogenic oocytes, and (iii) appearance of progressively enlarged vacuoles and degraded products; for instance, the vacuoles occupied 15% of the ovary of the females treated at 1000 μg/l. These degenerative changes resulted in the pronounced loss of oocytes. Consequently, the generation of critical minimum number of, say, 75 eggs, 'ready to spawn' required longer intervals between successive spawnings. However, vitellogenesis was not inhibited even in the females treated at 1000 μg/l, as all the recognizable oocytes were vitellogenic.

Male

On the 110th dph, the control male attained sexual maturity and began to build a bubble nest. However, the age at which the matured males immersed at 750 and 1000 μg/l began to blow the bubble nest was postponed; but, males treated at 500 μg/l attained sexual maturity on the 91st dph itself and regularly built the bubble nest once every sixth day (Table 5). The period between successive nest building activities also extended from 5 days in the control to 28 days in males treated at 1000 μg/l.

The male fighting fish builds bubble nests and cares for the fertilized eggs, sometimes even to a maximum period of 3–4 dph, the period during which a hatchling draws its nutrition from the yolk. Hence the area available within the bubble nest is critically important, and limits the number of fertilized eggs guarded during embryogenesis. Interestingly, not only the period between successive nest building activities was significantly extended from 5 days in the control to 28 days in males treated at 1000 μg/l, but the bubble nest area also decreased from 39 to 26 cm^2 (Figure 3). Briefly, the number of eggs guarded in the nest decreased from 150 in the control to 84 in the nest built by a male previously treated at 1000 μg NE/l.

Histology

Many degenerative changes were observed from the sections of testis of males, which were previously exposed to different NE doses. The most apparent were the following: (i) the obvious reduction (Figure 2 *f*) or almost virtual disappearance of spermatogonia (Figure 2 *h*); (ii) shrinking of the seminiferous tubule (Figure 2 *g*); (iii) a remarkable decrease in the density of spermatids (Figure 2 *h*), and (iv) 30% increase in vacuolar area in the testis of fish treated at 1000 μg/l (Figure 2 *h*).

Discussion

Our objective was to explore the use of NE to ensure masculinization in the ornamental fighting fish, but with-

out suffering reduction in body length and mortality at the end of treatment. Even the shortest cumulative period of 9 h discrete immersions of the hatchlings at a concentration of 900 µg/l MT results[3] in 24% mortality at the end of treatment and reduction in body length from 6 to 3.5 cm. This stunted growth led to 26% reduction in egg production. Kavumpurath and Pandian[5] administered four androgens through diet supplementation, and reported 36% mortality when the fighting fish was treated at the respective optimal doses of MT and NE. Discrete immersions for the shortest cumulative duration of 9 h at a dose of 1000 µg NE/l resulted in 29% mortality at the end of the treatment, but ensured normal growth and 92% masculinization. Hence NE may be chosen for masculinization of the fighting fish in the ornamental fish industry.

Another objective was to know whether an air-breathing fighting fish escaped to the surface when immersed in water containing a selected steroid. On being exposed to either NE or endosulfan[15], the two-day-old fighting fish hung to the surface of the water column. However, it began air-breathing on the fifth dph, and commenced regular air-breathing on the eighth dph. Apparently, the air-breathing labyrinthine organ developed between the second and fifth dph. Vivekanandan[13] traced the ontogenetic development of surfacing behaviour in the obligately air-breathing murrel *C. striatus*, and showed that the fish did not come to the surface on hatching, but began to regularly breathe air when it attained a body weight of 750 mg.

Except at the sub-optimal dose of 750 µg NE/l, the air-breathing frequency of the fighting fish was suppressed, in the five, eight and 180-day-old fighting fish, which were previously immersed at the tested concentrations of NE. Hence, the tested concentrations of NE suppressed the metabolism, as indicated by the air-breathing frequency which is regarded as an index of metabolism[16]. Conversely, Arunachalam and Palanichamy[17], reared the obligate air-breathing anabantid *Macropodus cupanus* in water containing sub-lethal concentrations of 1–2.5 ppm carbaryl, a widely used pesticide, and showed that the air-breathing frequency was significantly increased. Hence investigations in this area may yield interesting information on the 'trade-off' between escape from a pollutant and/or metabolic suppression by it.

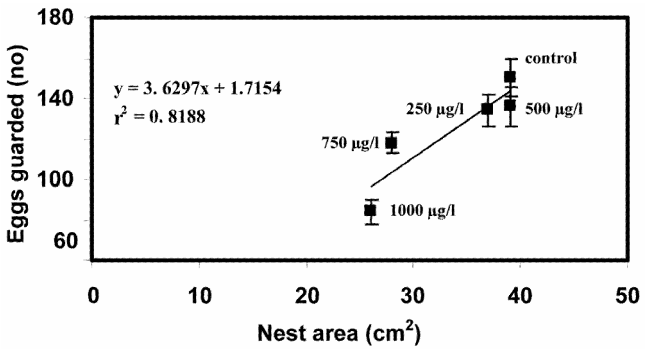

Figure 3. Effect of different doses of NE on the bubble nest area built by the fighting fish to guard the fertilized eggs.

Apparently, the chosen steroid (NE) influenced the growth of the treated fighting fish in different modes (in males and females) and at different intensities. Many steroids accelerated growth at the early stages, when the fish was treated at the sub-optimal dose. However, as the age of the treated fish advances, the accelerated growth is normalized[2]. For instance, George and Pandian[18] estimated the growth of *Poecilia sphenops*, treated with different doses of MT at the age of 3, 6, 9, 12, 15 and 18 months. Relative growth was enhanced in 3-month-old treated individuals with increased steroid dose up to pre-optimal level for sex reversal, beyond which the increase in relative growth began to diminish. Notably, growth was consistently suppressed in 18-month-old individuals, irrespective of the treatment intensity. Incidentally, it is also known that a chosen steroid, which ensures 100% sex reversal in a fish treated at the optimal dose, may induce paradoxical sex reversal in the same fish, when treated at super-optimal doses[19]. Pandian and Koteeswaran[20] have suggested a model in which the activity of aromatase, a 'turn-key' enzyme in sex differentiation, when fully expressed, reduces the normal androgenic profile in genetic males and results in the production of 100% female progenies. Alternatively, the reduction of its activity maintains the normal endogenous profile in the genetic females and results in the production of 100% male progenies.

Our observations on the zebrafish, *Danio rerio* immersed in endosulfan or tributyltin chloride, have emphasized the need for reproductive behavioural assay as an important component in assessing the reproductive toxicology of fishes[15]. Nash *et al.*[7] undertook a long-term experiment and showed that in the presence of ethynylestradiol-treated male, a treated female zebrafish produced eggs that were not viable. Studies on the Japanese medaka, *Oryzias latipes*[21–23] and the fighting fish[24] also indicate the importance of reproductive behaviour in assessing progeny production. The present study has shown that the NE-treated male fighting fish could not only induce the female to spawn as many eggs as that of the control male, but was also unable to accommodate those many fertilized eggs within the limited space of the smaller nest built by it. The relationship between eggs guarded and nest area was linear and significant. Our observations confirm the earlier report by Jaroensutasinee and Jaroensutasinee[10], which shows a similar relationship between the number of fertilized eggs and bubble nest area built by normal fighting fish of different body sizes. However, NE proved to be milder toxicant than endosulfan[15]; fighting fish treated at 1000 µg NE/l built a bubble nest with an area of 26 cm^2 and guarded >84 eggs, whereas even at 1000 times lower concentration (1000 ng/l) of endosulfan, the treated fighting fish built a bubble nest with an area of 15 cm^2 and guarded 56 eggs only.

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ACKNOWLEDGEMENTS. We thank the Indian National Science Academy, New Delhi and Madurai Kamaraj University, Madurai for financial support.

Received 22 April 2008; revised accepted 26 September 2008

Erratum

Radiation effects, nuclear energy and comparative risks

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[*Curr. Sci*., 2007, **93**, 1230–1248]

Page 1237, col 1, line 9 from the bottom: 3.13×10^{3} *should read as* 3.13×10^{10}
Page 1237, col 1, line 10 from the bottom: 1.6×10^{-6} *should read as* 1.6×10^{-13}
Page 1237, col 1, line 18 from the bottom: 1000 tonnes *should read as* 3 tonnes
Page 1244, col 1, line 5 from the top: 1.5–2 million tonnes *should read as* 0.75–1 million tonnes
Page 1245, col 4 in Table 3, 0.006 *should read as* 0.06 (note that for calculations in Appendix 1 the correct value of 0.06 is used).